Potential of exogenous L-amino acids in salinity stress alleviation during germination and early post-germinative seedling growth of *Lactuca sativa* L.

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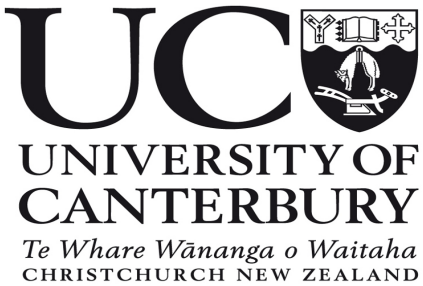

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Abstract

Soil salinity is a common abiotic stress for plants, that is having an increasing impact on international food production. A practical strategy to help mitigate the adverse effects of salinity stress on crop productivity is to increase salt tolerance of crop plants. It has been shown that exogenous application of L-proline and L-glutamate is capable of reducing the severity of salinity stress on seed germination and early seedling growth of brassica and cucumber, respectively. The main aim of the present study was to investigate the potential of all 20 common protein amino acids to alleviate salinity stress in lettuce (*Lactuca sativa* L., variety 'Great Lakes') during and immediately following germination. Sowing lettuce seeds in different concentrations of sodium chloride (NaCl) adversely affected germination and early seedling growth in a dose-dependent manner. After 48 hours of sowing lettuce seeds in 1 mM of any of the 20 exogenous amino acids in the absence of NaCl, it was found that the amino acids also inhibited seedling growth, particularly root elongation. However, in direct treatment experiments involving addition of seven amino acids singly (L-asparagine, L-isoleucine, L-leucine, L-proline, L-phenylalanine, L-tyrosine and L-valine) to an inhibitory concentration (60mM) of NaCl, it was found that lettuce seedling growth was protected from the salt stress. Additionally, seeds pre-treated for 8 hours before germination with L-arginine, L-glycine, L-histidine, L-methionine and L-phenylalanine, showed significant growth recovery after a further 40 hours growth exposed to 60mM NaCl.

The measurements of cell size in root maturation zone and mitotic index at the root tip of lettuce seedlings after 48 hours from sowing seeds suggested that it might be possible that some amino acid treatments could affect cell elongation and / or cell division. However, further in-depth investigations are required and warranted to elucidate the mechanism(s) whereby exogenous amino acids could play a role in alleviation of salt stress in lettuce. It is concluded that several L-amino acids have the potential in pre-sowing seed treatment (seed priming technology) to enhance salt tolerance for crop stand establishment in soils with salinity issues.

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Introduction

1a. Multiple Biological Functions of Protein Amino Acids

The 20 protein, or standard, amino acids serve as the structural units of proteins. Besides this core role in cell maintenance, they serve many alternative roles in cellular metabolism, in particular as metabolic intermediates for a range of vital molecules (Sharma and Dietz 2006; Garcia, et al. 2011). In plants, protein amino acids often have a role in hormonal interactions, and regulating growth and development (Woodward and Bartel 2005; Galili et al. 2008). While amino acids are both extremely abundant and important, many aspects of their role in plant metabolism and signalling remain unknown. The relationship between amino acids and stress response is one of increasing interest. Protein amino acids have also been found to play a role in metabolic responses of plants to abiotic stress. In particular, L-proline has been found to be linked to several abiotic stresses in a number of studies on multiple plant species (Nawaz, et al. 2010; Szabados and Savoure 2010). There has, in recent decades, been a lot of work to further explore the effects of externally applied amino acids, and identify what benefits they may offer in agricultural production.

Several papers have investigated the role of amino acids in soil solutions as a basic nutrient (organic nitrogen) source. While microbes are historically thought to sequester the majority of available soil amino acids, there is some evidence in recent years that plants are capable of uptake and utilisation of amino acids as an organic nitrogen source as well (Lipson and Nasholm 2001). It has been shown that many of the available amino acids are readily taken up by *Arabidopsis thaliana (*L. Heynh), and it appears that only metabolic restrictions limit the use of amino acids as N sources by plants (Forsum, et al. 2008). In *Arabidopsis* exogenous application of a few L-amino acids, particularly L-glutamine, L-asparigine, L-aspartic acid, L-glycine, L-arginine and L-alanine led to increased biomass in the absence of an alternative inorganic nitrogen source, suggesting *Arabidopsis* is able to utilise these amino acids (Forsum, et al. 2008). This benefit was unique to L-amino acids. D-enantiomers of the relevant amino acids were found to be taken up at a lower rate than the L-enantiomers, and were not utilised metabolically in plants (Forsum, et al. 2008).

The findings on the effects of exogenous amino acids on plant growth are somewhat controversial , however. Earlier studies concluded that, apart from L-glutamine, all other protein amino acids were toxic to plants (Bonner, et al. 1996). Addition of L-glutamine was found to

promote plant growth by acting as an effective nitrogen source or supplement, and in combination with other amino acids, was capable of alleviating their inhibitory effects (Bonner, et al. 1996). In addition there is some evidence that exogenous amino acids may promote plant growth through their involvement in other metabolic processes and as signalling molecules, rather than simply as a nutrient source (Walch-Lin, et al. 2006). Several roles have been identified that contribute to plant stress response. The mechanism whereby an amino acid could mediate stress-relief appears to differ between different amino acids. Some previously studied amino acids also seem able to help plants to respond to a range of stresses through different mechanisms (Gzik 1996; Sharma and Dietz 2006; Szabados and Savoure 2010). Much of the research in this area focusses on a specific amino acid, rather than the performance of a range of amino acids.
L-glutamate has been recently linked to growth promotion in salt-stressed cucumber, through interaction with the plant hormone ethylene (Bonner, et al. 1996; Chang, et al. 2010). The effect of L-proline, in particular, has been the focus of a great deal of research (Nawaz, et al. 2010; Szabados and Savoure 2010).

1b. L-Proline

L-proline is an essential amino acid that is produced by all plant species (Wani, et al. 2012), and plays a role in a range of metabolic functions. Its role in plant response to abiotic stresses has been examined in a number of experiments in recent years and among all amino acids it is now the best understood regarding its relationship to stress response (Gzik 1996; Szabados and Savoure 2010).
Endogenous levels of L-proline have been shown to increase significantly in plants under a wide range of stresses (Kaul, et al. 2008; Szabados and Savoure 2010) . In particular, L-proline is found to play important roles in plant response to heavy metal stress (Sharma and Dietz 2006), and osmotic stress. For example, L-proline has been found to function as an osmoprotectant, and plants overexpressing a carboxylate synthetase gene involved in L-proline production can develop drought resistance (Kavi Kishor, et al. 1995; Nawaz, et al. 2010).
In many studies, increases in endogenous L-proline levels in stressed plants were accompanied by increases in growth compared to control plants with lower L-proline production.

Application of exogenous proline is also able to promote resistance to certain stresses (Nawaz, et al. 2010). Plants under some forms of osmotic stress, such as high salinity or drought stress, are often known to produce reactive oxygen species (ROS) which could result in cell injuries and even death (Munns 2005).

The effect of L-proline in stress response does not appear to be universal. Many older studies investigating a range of amino acids did not identify exogenous L-proline as having a positive growth effect. In unstressed plants, application of proline has even been found to have a negative impact on plant growth (Bonner et al. 1996). This implies the presence of species-specific responses, even from common and relatively well understood amino acids.

1c. Salinity stress

Salinity stress is a common environmental stress for plants. High salinity in soils can have a hugely detrimental effect on plant growth, and the economic yield of many crop species can be severely curtailed due to rising salinity in regularly irrigated soils (Rengasamy 2006).

Throughout the world, a huge area of land is becoming unsuitable for crop production due to rising salinity issues. While the majority of this is a result of natural salinity levels, (Munns 2005) incidence and severity of secondary salinity is increasing all over the world.
Secondary salinity is defined as that which results from human activities, usually through agricultural impact, or regular irrigation (Munns 2005; Rengasamy 2006). Soil salinity can also be greatly increased by seawater intrusions, such as tsunamis, or regular coastal flooding. Events such as these can have a huge long term impact on the nutrition and economy of a region, especially in poorer areas, such as those situated in South East Asian Deltas.

Cultivated land with high food production capacity can also experience substantial changes in soil salinity. Repeated irrigation with poor quality water results in substantial increases to soil salinity levels, and rapidly decreasing crop yield and quality. Issues such as this pose dire threats to global food sustainability; irrigated land has twice the food productivity of rain fed, giving a disproportionately large impact on global food production (Munns 2005). Future increases in food production are primarily dependant on increasing yield from currently utilised land, rather than utilisation of different land areas. This makes maintaining and increasing the quality of soil in cultivated areas of utmost importance.

Detrimental soil salinity can be combated by either lowering the salinity levels in the soil, which is a time consuming and costly process, or increasing the ability of crops to survive in a high salt environment. As mentioned above, there are a few amino acids that have known stress-relief properties. Further investigation into the mechanisms behind this activity will provide valuable

information about techniques to improve salt tolerance in important crops. Further study of other amino acids is likely to identify further avenues for this type of research.

1d. Lettuce (*Lactuca sativa* L.)

Lettuce (*Lactuca sativa* L.) is a fast germinating crop plant. It is one of the most commonly used salad vegetables, and is of high economic importance (Kim et al. 2008). Due to rapid growth and no lag time, lettuce is capable of germinating in ideal conditions in less than 12 hours, which has made it an ideal model plant for germination experiments. Lettuce is also able to germinate in a range of light and temperature conditions; this means experiments can be conducted easily, without the need for specialised growth rooms. Due to the relatively high number of treatments in this experiment, the ability to quickly and reliably germinate seedlings was important.

Many lettuce cultivars, including the Great Lakes variety used here, exhibit low salt tolerance compared to many other common model species (Kim et al. 2008). This makes it very easy to establish and observe the effect of salinity, supplied as sodium chloride (NaCl), on seedling growth.

1e. Germination and post-germinative seedling growth

Seed germination is arguably one of, if not the, most important stages in a plant's life cycle, due to it often being a time of particular vulnerability as a result of resources being diverted from protectionist systems to growth (Nasri et al. 2011). A high percentage of seed germination and vigorous post-germinative seedling growth is crucial for maximal crop establishment, ensuring production of high yields. As such, stresses experienced by seeds during germination can have a significant negative impact on the seedling vigour and subsequent crop yield, and therefore economic potential of a crop. Several studies have also identified differing responses between germinating or developing plants versus mature plants growing in a stressful environment (Zapata et al. 2004). This makes the preservation of seedling vigour under stressful conditions an important aspect of crop production. In light of this, a great number of previous studies have examined the impact and mechanisms of many stresses experienced during seed germination, as well as methods to lessen their impact (Zapata et al. 2003; Nasri et al. 2011) .

1f. Seed priming

Seed priming is a technique by which seeds are treated before seed germination, to aid plant development following germination. Seed priming has been used in the past for a range of applications. It is commonly used to promote resistance to particular stresses, or to circumvent dormancy periods to promote uniform crop germination (Cantliffe et al. 1984; Farooq et al. 2008; Mahmoudi et al. 2012) . For example, seed priming with salicylic acid has been used to reduce effects of thermodormancy in lettuce (Cantliffe et al. 1984). This practice is most often used with plant hormones or other common endogenous growth regulating compounds.

In this project, seed priming with amino acids was investigated. This might allow seeds pre-treated with L-amino acids to gain beneficial effects, including retaining a growth or stress resistance benefit following and during germination. This can allow for batch treatment of seeds, which can then be stored or shipped until germinated, rather than needing to treat seeds as they are germinated. Results from experiments such as this can also supply additional information about the way amino acids can influence seedling growth, and interact with salinity stress.

1g. Ethylene

Ethylene is a major hormone in plants. The association between ethylene and seed germination has long been established. However the exact nature of its role in germination is not fully understood (Zapata et al. 2003; Zapata et al. 2004).
Ethylene is also thought to be heavily involved in stress response in a number of plant species. Previous studies have shown that ethylene production increases in many plant species, including lettuce, under salinity stress. Some investigations into lettuce seedling response to salinity stress have found that ethylene production is further increased in salt tolerant cultivars, compared to more susceptible varieties (Zapata et al. 2003). This is an effect that has also been observed in rice grown in saline conditions. However, in other species, including wheat, the opposite interaction has been identified; salt-sensitive cultivars are found to accumulate more ethylene than salt-resistant ones. This strongly implies again that this is a system that can vary greatly between different plant species. However, in either case it is clear that ethylene is closely related to stress-response in a range of plant species. Further studies into this relationship will likely produce valuable knowledge about the mechanisms, including the involvement of amino acids, of plant stress response.

1h. Aims, Hypotheses and Experimental Approach

There is a wealth of literature about the actions and relationships of specific amino acids with different plant species and different abiotic stresses. However, in many cases separate studies have produced conflicting conclusions regarding the effect of an amino acid under some experimental conditions. Studies of this nature have also all focussed on the activity of a single or only a few amino acids. The majority of these studies have also been carried out on mature, or partially developed plants. While there are a few studies examining the effect of amino acids on germination specifically, the influence of most of the amino acids on early seedling development is poorly understood.
Given these previous findings, the primary aim of this study was to examine the impact of each of the 20 protein L-amino acids on germination and early post-germinative seedling growth in lettuce, both under normal growth conditions and environmental stress, in the form of a growth-inhibiting NaCl solution.

It is hypothesised that several L-amino acids will have a positive impact on seedling growth under salt stress. In particular, L-proline and L-glutamate are expected to produce a positive result, in line with the findings in previous studies. In addition, it is hypothesised that at least a few other amino acids may also exhibit a positive effect in salt-stressed plants. It is also possible that there are a number of amino acids that have a positive effect on plant growth in unstressed conditions. It also seems likely that amino acids will act in stressed plants in a specific stress-reducing capacity, while in unstressed plants any promotion will be due primarily to general nutrient effects, such as functioning as a supplementary organic nitrogen source. As a result of this, it is expected there will be some amino acids capable of providing stress-relief which do not promote seedling development generally.

The efficacy of pre-treating seeds will also be investigated. Successful seed priming through pre-treatment may contribute to development of an economically viable and easy way to distribute salt-resistant seeds. While the effects of pre-treating should be in line with directly treating seeds, any results obtained will further contribute knowledge about the mechanisms behind plant stress response.

The primary aim of this study was to fully investigate the impact of all 20 protein amino acids on seedling growth and plant stress response in lettuce, focusing on the role and effect of amino acids during and shortly after germination. Previous studies have almost exclusively focussed on

a single, or a small selection of target amino acids, leaving large gaps in our understanding. Many of the results obtained in previous studies are also inconsistent or contradictory, particularly regarding the role of L-amino acids in stress response. This study, therefore, undertakes a comprehensive analysis of the effects of all the protein amino acids on growth and salinity stress response. Additionally, it focuses on germination, an extremely important phase of plant development in relation to plant productivity, and one which has been irregularly studied in this context, and is therefore relatively poorly understood.

1I: Nomenclature and key

In certain areas of this thesis, L-amino acids may be referred to by their 3-letter code. These are as follows;

Ala – alanine
Arg – arginine
Asn – asparigine
Asp – aspartate/aspartic acid
Cys – cysteine
Gln – glutamine
Glu – glutamate/glutamic acid
Gly – glycine
His – histidine
Ile – isoleucine
Leu – leucine
Lys – lysine
Met – methionine
Phe – phenylalanine
Pro – proline
Ser – serine
Thr – threonine
Trp – tryptophan
Tyr – tyrosine
Val - valine

Methods and Materials

Exogenous L-amino acids were applied in two different types of experiments (direct treatment and pre-treatment experiments) to evaluate if any of the 20 L-amino acids might influence lettuce seed germination in the presence or absence of a high concentration of NaCl. The details of these experiments are described separately later.

2a. Plant material

Lettuce seeds (*Lactuca sativa* L. variety 'Great Lakes') were purchased from Kings Seeds (Katikati, New Zealand) and were stored within their original aluminium packaging in a fridge kept at 4°C.

2b. Chemicals

All amino acids used in this study were obtained from Sigma (St. Louis, Missouri, USA). Sodium chloride and other chemicals are of reagent grade from BDH (UK).

For salt treatments, first a 0.6 M sodium chloride (NaCl) stock solution was prepared by dissolving 17.5 g NaCl in 500 ml of de-ionised water. For treatments with amino acids, the concentration of the stock solutions of different L-amino acids was 10 mM except that of L-tyrosine. To make up the 10 mM stock solutions of 19 L-amino acids, 500 umol each of the selected amino acid was dissolved in 50 ml of de-ionised water. L-tyrosine was prepared slightly differently. At the relevant stock concentration (10 mM), L-tyrosine did not dissolve in water at room temperature. After boiling L-tyrosine was dissolved, but it was found to be precipitated out of solution during storage at room temperature within 24 h. In order to overcome this problem, L-tyrosine was made up as a 1 mM solution and used in experiments without further dilution.

2c. Influence of exogenous amino acids on lettuce seed germination: A preliminary survey

To investigate the effect of each of the 20 common L-amino acids on lettuce seed germination, 5 ml of 1mM L-amino acid, or de-ionised water (control), were placed into a clear 35 ml plastic tube in duplicates (ThermoFisher Scientific, New Zealand) for each of the 20 L-amino acids. Ten seeds were then sown on the surface of the solution in each tube. The seeds were left to germinate and grow for 24, 48 or 96 h in a growth room in the dark at 25^0C, before the percentage of seeds germinated was recorded and the germinated seedlings were removed for closer visual inspection and comparison to control. Since the purpose of this initial experiment was to investigate the

general response of lettuce seedlings to each amino acid applied singly during germination, only general growth trends, rather than specific measurements, were recorded.

2d. Effect of sodium chloride on lettuce seedling growth

Lettuce seedling growth in the presence of sodium chloride (NaCl) was examined without the addition of any exogenous amino acids. The purpose of this experiment was to establish a baseline response of lettuce seedlings to an environmental stress factor.
Ten seeds were sown on the surface of 5 ml of varying concentrations (30, 60, 90 or 120 mM) of NaCl, or de-ionised water as control in a 35 ml clear plastic tube (ThermoFisher Scientific, New Zealand), as shown in *Figure 1* below, and left to germinate and grow for 48 h in a growth room in the dark at 25^{0}C . Seedlings were then removed and the growth parameters (hypocotyl length, radicle length and overall seedling length) were measured using the method described below. The percentage of seeds germinated was also recorded in this experiment. There were four replicates in each treatment comprising a total of 40 seedlings and the data obtained for to each parameter measured was used to determine a mean value and S.E.

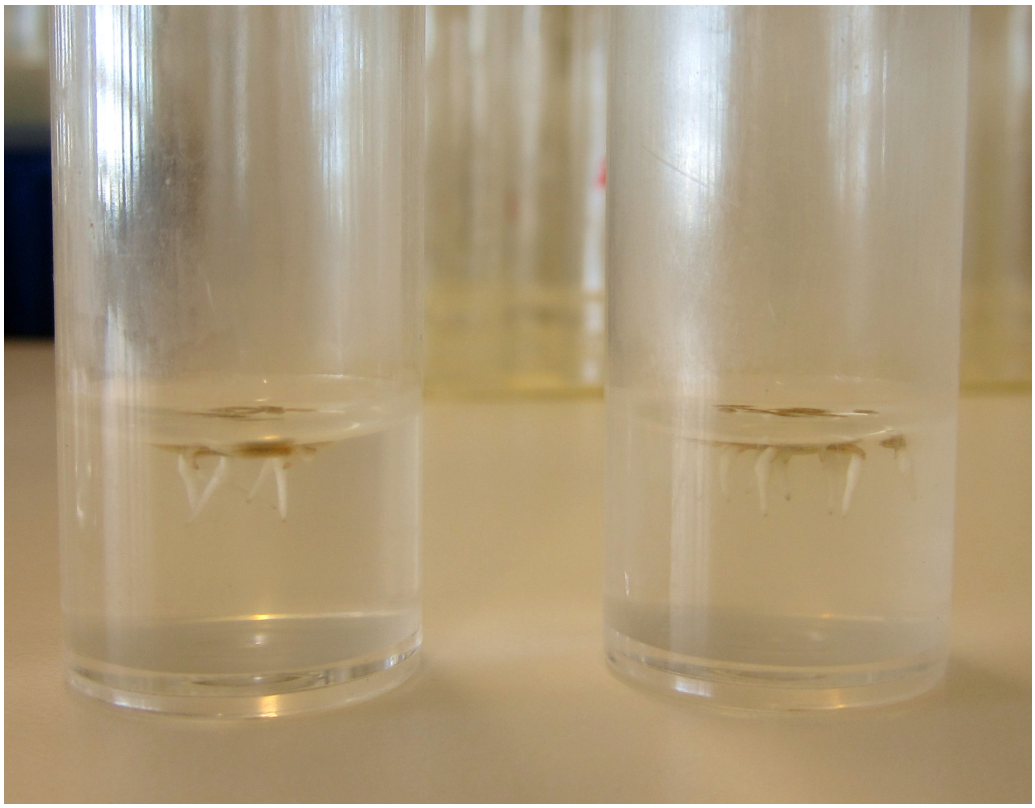

Figure 1. Lettuce seedlings grown from seeds sown on the surface of a test solution (5 ml) in a growth room in the dark at 25^{0}C. This photograph was taken 48 hours after seeds were sown.

2e. Direct treatment experiments

Ten seeds were sown on the surface of 5 ml of a test solution comprising either: (a) 1mM of an L-amino acid, (b) de-ionised water as control, (c) 60mM NaCl solution, or (d) 1 mM of the selected L-amino acid in the presence of 60 mM NaCl, in a 35 ml clear plastic tube. Seeds were allowed to germinate and grow for 48 h in the dark at 25^{0}C . Seedlings were then removed and the growth parameters were measured. There were three replicate tubes in each treatment, and the experiments were repeated at least twice. Data from each treatment in all the repeated experiments were used to determine the mean and S.E.

2f. Pre-treatment experiments

One sheet of Whatman No. 1 filter paper was placed in one plastic Petri dish (9 cm diameter) per treatment and approximately 40 seeds were placed evenly on the surface of the filter paper in each. To one of the Petri dishes in each experiment, 5 ml of de-ionised water was added and to the others 5 ml of an L-amino acid solution. In a dark growth room at 25°C, seeds were imbibed in a treatment solution for 8 h without being submerged, as shown in *Figure 2* below.

Following this treatment period, the seeds treated with an amino acid were divided into four batches per petri dish, each consisting of ten seeds. Two batches were transferred separately to two 35 ml plastic tubes each containing 5 ml of deionised water (control). Similarly, the two remaining batches were transferred to two 35 ml plastic tubes each containing 5 ml of 60 mM NaCl. For comparison, the seeds treated with de-ionised water were also divided into four batches and were similarly transferred to tubes containing either de-ionised water or 60 mM NaCl. Then all the tubes were kept in the same growth room at 25^{0}C for 40 h before the seedlings were removed and hypocotyl and radicle lengths measured.

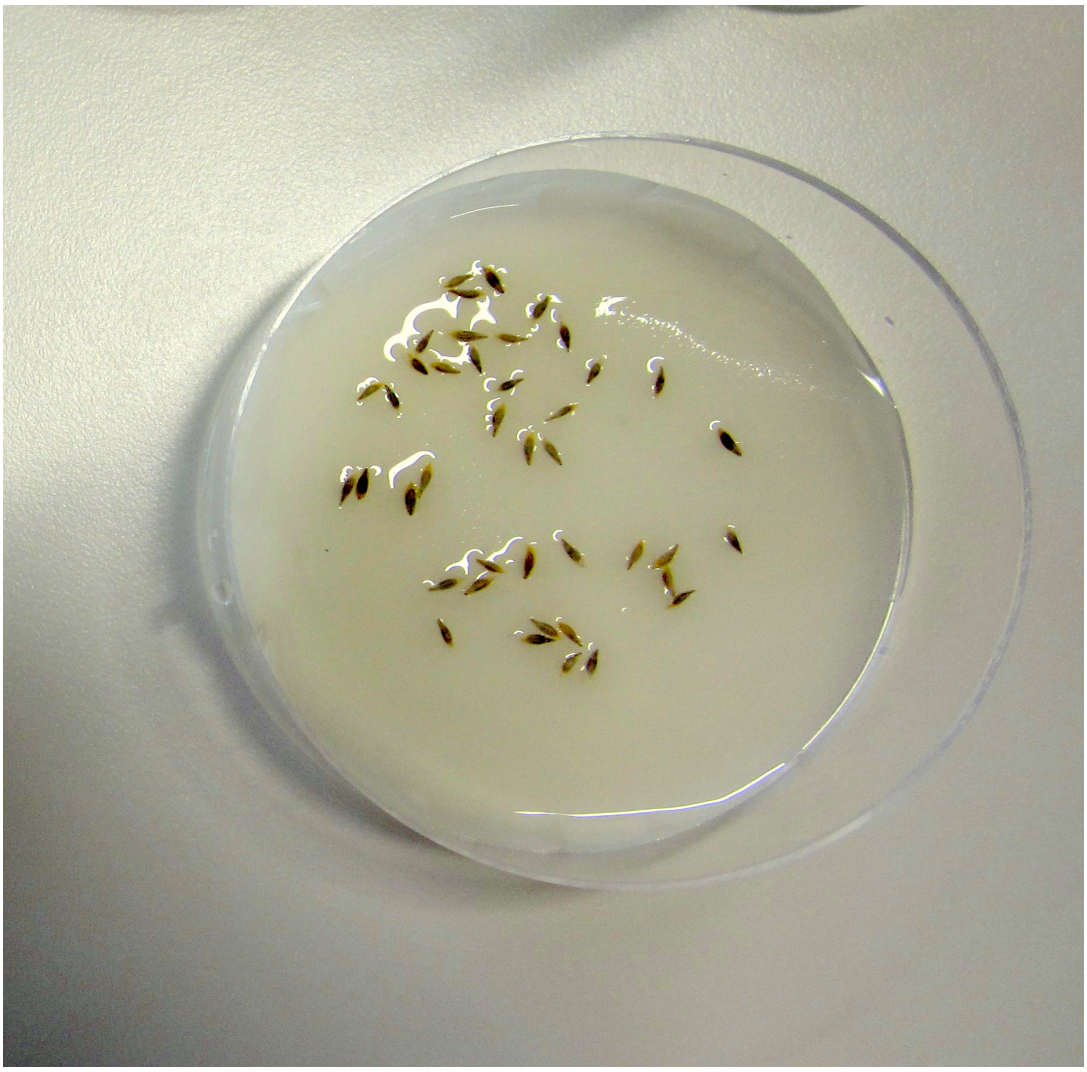

Figure 2. Example of seeds sown on the surface of a sheet of Whatman no.1 filter paper moistened with a test solution in a Petri dish during a pre-treatment experiment.

2g. Growth Measurements

Due to the relatively small size of lettuce seedlings, measuring the length of seedling parts using a ruler would not be precise enough, and was deemed impractical. Instead, the length of seedling parts was determined with the help of high resolution photos from which pixel counts were made using a photo-editing software (Microsoft Paint, Windows 7).

After growth in a test solution, seedlings were arranged in a row and photographed on a piece of 5 mm graph paper, as demonstrated in *Figure 3* below. A simple photo editing program, such as Microsoft Paint, was then used to determine the size of the graph squares in pixels. This gives a pixel to mm ratio for each photo/dataset. From this, a simple calculation was used to convert the

length of a seedling, or its parts, in pixels into mm using the following formulae as below:

$$[p/m] = s(px)/5$$

$$p(mm) = s(px)*p(px)$$

$$p(mm)=p(px)/[p/m]$$

Where; s(px)= The number of pixels in a square.
[p/m]= The number of pixels per mm
p(px)= The size of the measured seedling or organ, in pixels
and p(mm)= The size of the seedling in mm

This method allows for the accurate measurement of growth differences of a few millimetres among lettuce seedlings during early post-germinative growth.

2h. Treatments under controlled pH conditions

A 0.1 M solution of MES (2-(*N*-morpholino)ethanesulfonic acid) was made up in deionised water, and pH was adjusted to 5.6 using 0.1 M NaOH. This buffer was then used in place of de-ionised water to repeat the direct treatment and pre-treatment experiments to account for any differences in growth effects that might result from possible pH variations of the L-amino acid test solutions. The pH of the test solutions after mixing an appropriate aliquot of a selected L-amino acid stock solution with the MES buffer was tested again before seeds were sown to ensure the pH of the resultant test solution was maintained in the presence of the L-amino acids. The effects of amino acids of interest were also investigated using pH controlled test solutions. L-isoleucine, L-leucine and L-phenylalanine in direct treatment experiments, and L-asparigine, L-aspartate, L-glycine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-serine, L-threonine and L-valine in pre-treatment experiments, were tested.

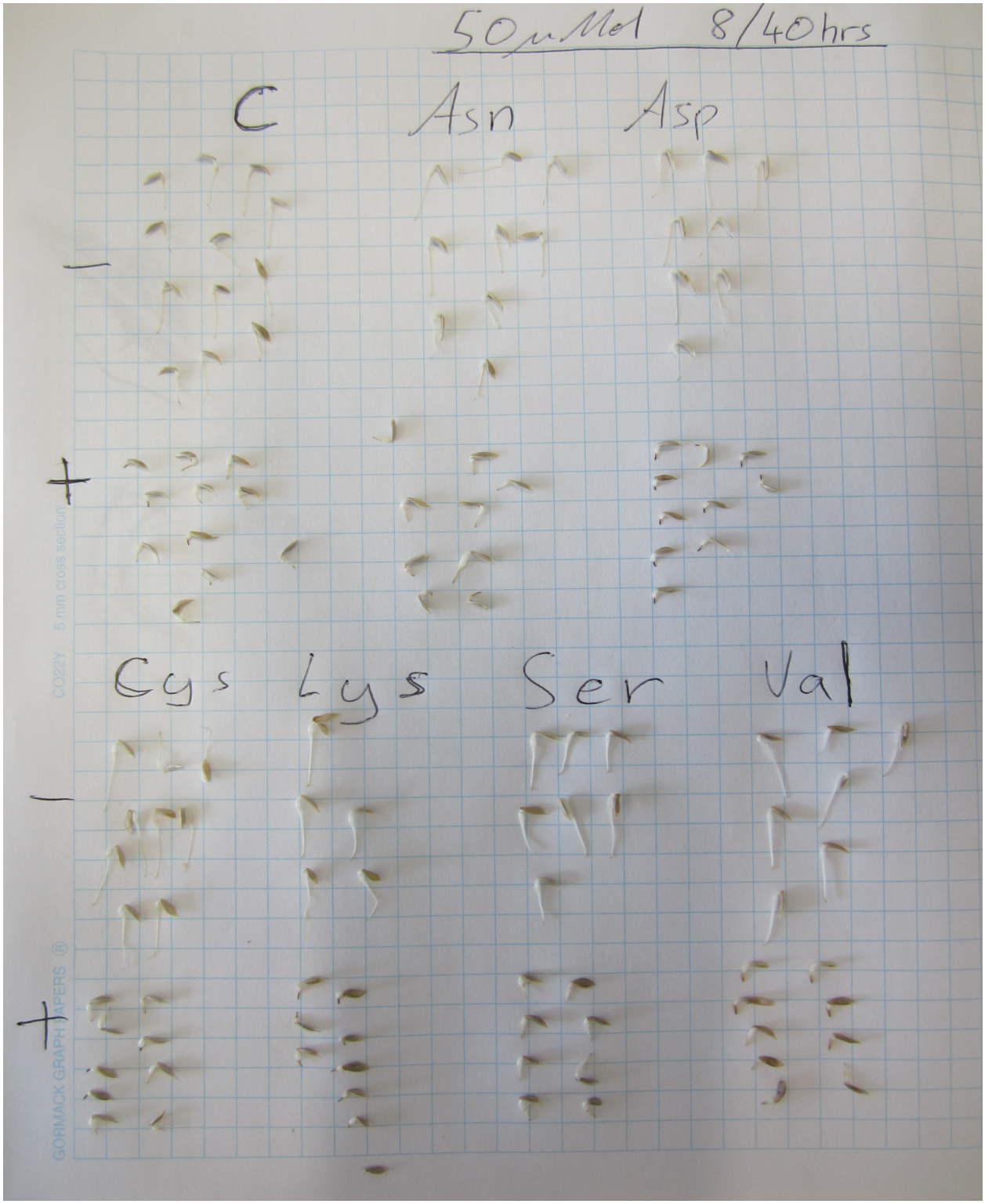

Figure 3. Seedlings following 8 hours pre-treatment with 1mM L-amino acid, then 40 hours in de-ionised water or 60mM NaCl solution. Seedlings were arranged by L-amino acid treatment, and – or + NaCl treatment, then photographed and sizes digitally measured.

2i. Treatments with multiple amino acids

Some of the L-amino acids of interest were tested in combination to identify any possible interactions between effective exogenous amino acids. These tests were conducted using the direct treatment procedure only. The following combinations of amino acids were tested: L-isoleucine / L-leucine, L-isoleucine / L-phenylalanine, L-leucine / L-phenylalanine and L-isoleucine / L-leucine / L-phenylalanine. For each experiment, the relevant L-amino acids were combined to give a solution with a total combined L-amino acid concentration of 1 mM. Therefore, for the L-amino acid pair tests, 2.5 ml of 500 uM of each L-amino acid were combined. For the triplet combination, 1.67 ml of 0.3 uM of each of the 3 L-amino acids were combined to give the final solution. Ten seeds per test solution were sown as in the treatments when amino acids were applied singly.

2j. Cell Size

Relative cell sizes were measured using an Olympus C011 light microscope at 400x magnification. For each selected amino acid treatment, after 48 h of germination and growth in the absence or presence of 60 mM NaCl, five seedlings were first transferred into 5 ml of 9:1 ethanol:acetic acid for fixation for at least 24 h. Prior to examination under the light microscope, seedlings were removed from the fixation mixture and washed sequentially in 90, 70, 50 and 30% (v/v) ethanol, before being cleared in an 8:1:2 mixture (w/v/v) of chloral hydrate:glycerol:water (Ding et al. 2010)

For each seedling measured, the lengths and widths of 20 randomly chosen cells in the root hair region of the hypocotyl were measured with an ocular micrometer.

Measurements were taken at the longest point longitudinally to determine cell length, and the widest point in the case of cell width.

2k. Mitotic Activity

For each selected amino acid treatment, after 48 h of germination and growth in the presence or absence of 60 mM NaCl, seedlings were fixed in a mixture of 3:1 (v/v) ethanol:acetic acid for 12-24 hours. Before examination under the light microscope, seedlings were hydrolysed in hydrochloric acid (HCl). Multiple seedlings were placed in approximately 20 ml of 0.5M HCl at room temperature for between 12 and 24 h, and then washed in de-ionised water. Folowing this, root tips (each approximately 2-3 mm long) were excised and then stained in carbol fuchsin for 10-15

min, before dipping in de-ionised water. Effective staining time varied slightly among different amino acid treatments. Finally, root tips were mounted on a glass slide in an 8:1:2 mixture of chloral hydrate:glycerol:water, then squashed firmly under a cover-slip to spread the cells (Ding et al. 2010).
The stained section was then examined under the Olympus C011 microscope at 400x multiplication. Visibly dividing cells were counted, and the mitotic index was calculated from the number of dividing cells per field of view, consisting of approximately 1250 cells.
Three frames of view were examined per root tip, and those from at least 4 seedlings per treatment were examined.

2l. Data analysis

All analysis was carried out in the statistical package R (version 3.0.3).
In the experiments on the effect of varying NaCl concentrations, the results with a linear relationship were analysed using a simple linear regression in R, with significance calculated from a 95% confidence interval.

All other analyses were conducted regarding data from unrelated treatments versus control. All the data were assumed to have a normal distribution, and were analysed initially using a basic analysis of variance (ANOVA). Any significant difference was established using a 95% confidence interval. Any datasets demonstrating significance were further analysed using a Tukey's range (honest significant difference) test, to identify significant differences between specific individual parameters.

Results

3a. Effect of varying concentrations of sodium chloride (NaCl)

In de-ionised water (control), approximately 80% of lettuce seeds germinated after 48 h in the absence of NaCl. The same germination rate was maintained in the presence of both 30 and 60 mM of NaCl. However, at 90 mM of NaCl, successful germination rate was reduced to below 60%. Less than 30% of the seeds germinated successfully in the presence of 120 mM of NaCl after 48 h.

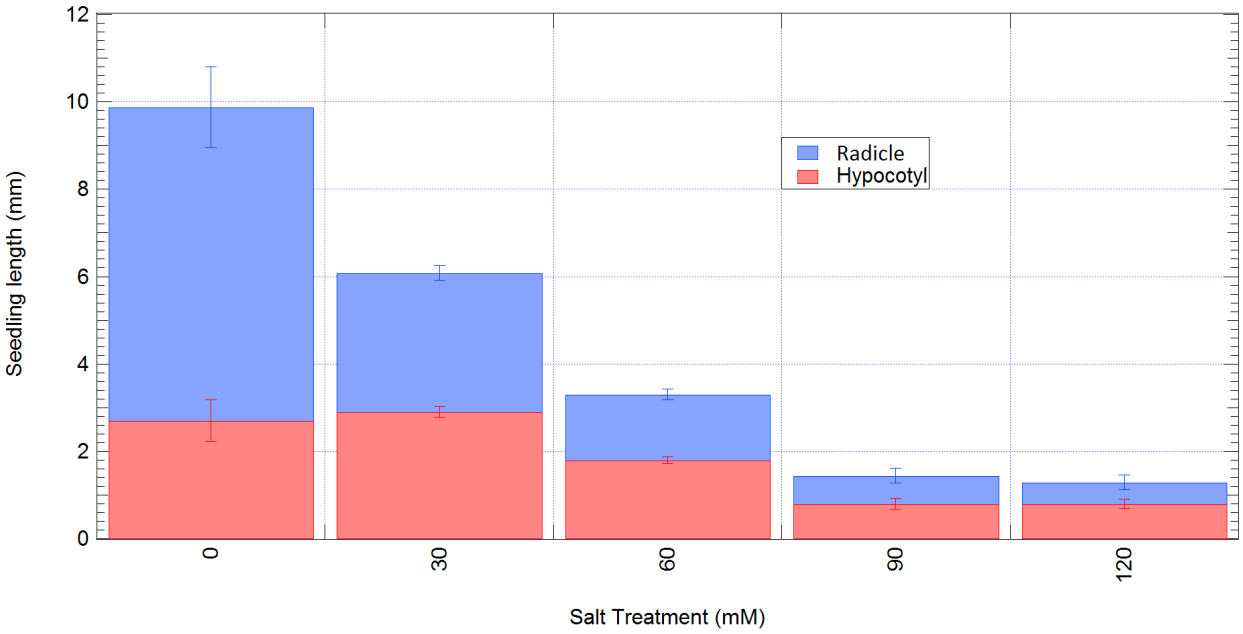

Figure 4: Demonstrating the effect of different concentrations of NaCl, or de-ionised water as control, on hypocotyl and radicle lengths 48 hours after seed sowing.

In the absence of NaCl, the length of the seedling was around 10 mm (Figure 4). This was decreased significantly with increasing concentrations (30 to 120 mM) of NaCl. Significance was calculated with a linear regression, giving a P value of 0.0186. In 30mM NaCl hypocotyl length was unaffected compared to control, with all growth inhibition occurring in the radicle (Fig. 4). At higher NaCl concentrations, hypocotyl growth was also inhibited. Interestingly, the ratio of the hypocotyl length to radicle length was increased in response to treatment with increasing concentrations of NaCl in a dose-dependent manner (Table 1). Similarly, hypocotyl length as a percentage of the total seedling length also showed this pattern of response to increasing NaCl concentration.

Table 1: Effect of different concentrations of NaCl on the ratio of hypocotyl length to radicle length of lettuce seedlings and hypocotyl length as % of total seedling length after 48 h from sowing seeds.

	NaCl Concentration (mM)				
	0	30	60	90	120
Total (mm)	9.88	6.08	3.31	1.44	1.29
Hypocotyl (mm)	2.7	2.9	1.8	0.8	0.8
Radicle (mm)	7.18	3.18	1.51	0.64	0.49
Ratio of hypocotyl length to radicle length	0.37	0.91	1.19	1.25	1.63
Hypocotyl length as % of total seedling length	27	48	54	55	62

3b. Direct treatment with amino acids on the length of seedling parts

In the absence of salinity stress

In the absence of NaCl, the effect of sowing lettuce seeds in 1 mM of exogenous L-amino acids was studied. None of the 20 common L-amino acids promoted lettuce seedling growth after 48 hours compared to the de-ionised water control treatment (Figure 2). Instead, each of the 20 L-amino acids inhibited early lettuce seedling growth by at least 20%. Many of the amino acids tested significantly reduced seedling lengths compared to those grown only in de-ionised water. Of particular note were L-aspartic acid, L-glutamate and L-tryptophan, all of which exhibited growth reduction of more than 85% compared to control seedlings.

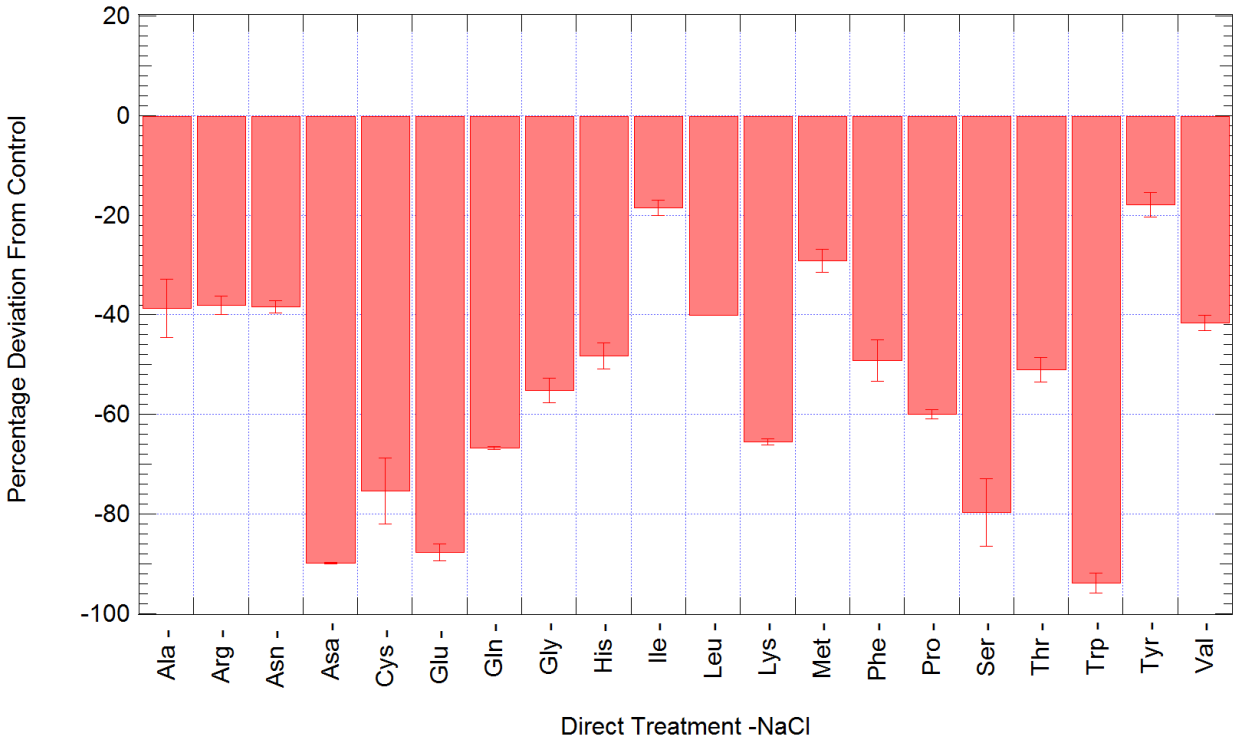

Figure 5: Seedling growth (length) following 48 hours treatment with each L-amino acid, shown as percentage deviation from de-ionised water control treatment. Error bars indicate standard error of the mean.

Hypocotyl length was found to be close to or the same as that of the deionised water control among 11 of the exogenous amino acid treatments: L-arginine, L-asparagine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryrosine, and L-valine (Figure 6). Decrease in hypocotyl length in response to exogenous amino acid treatment was particularly severe when overall seedling growth was <50% of control seedlings. Severe reduction in hypocotyl length was observed in the treatment of L-aspartic acid, L-glutamic acid, and L-tryptophan.

In contrast, radicle length exhibited inhibition in most of the exogenous amino acid treatments. The most severe inhibition of radicle length was found in the L-serine and L-tryptophan treatments, where radicle length was reduced by over 95% from control. The least inhibition (less than 50% inhibition) of radicle length was found in seeds treated with L-alanine, L-asparagine, L-isoleucine, L-methionine, and L-tyrosine (Figure 6).

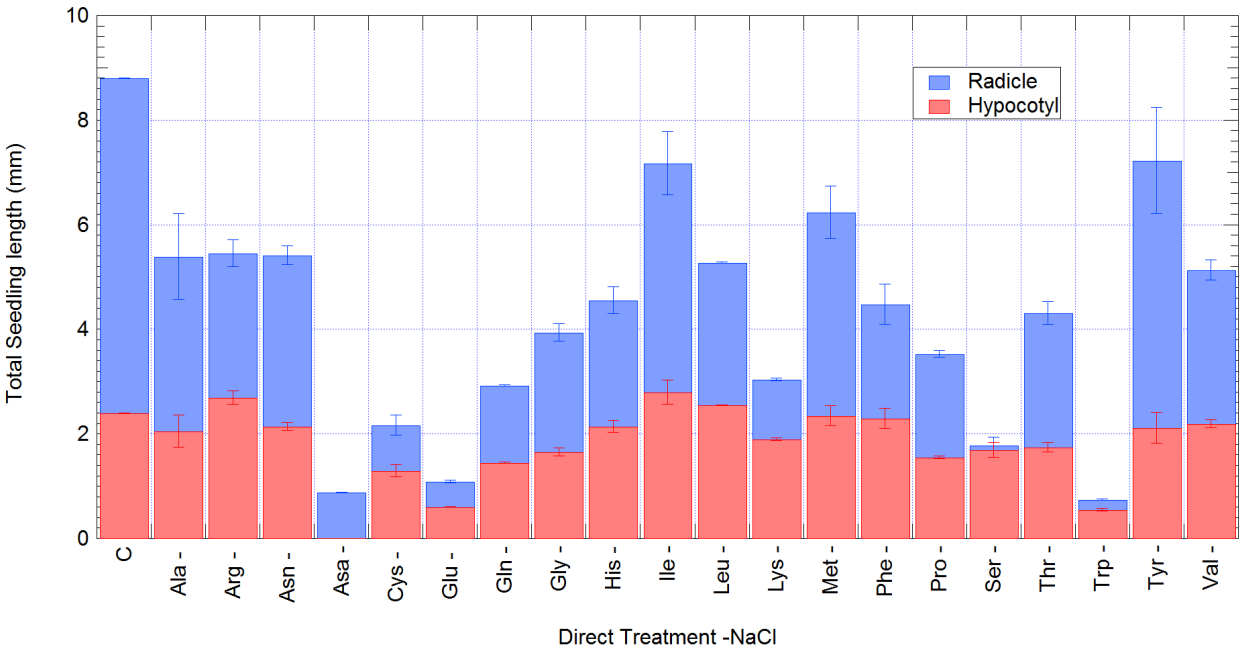

Figure 6: Hypocotyl and radicle length following 48 hours direct treatment with one of the 20 L-amino acids, or de-ionised water (control). Hypocotyl growth remains relatively constant between most treatments. Error bars indicate standard error of the mean.

Exogenous amino acid and salinity stress

In the presence of a detrimental level (60 mM) of NaCl, seven exogenous amino acids (L-asparigine, L-isoleucine, L-leucine, L-proline, L-phenylalanine, L-tyrosine and L-valine) produced a positive effect on lettuce seedling length compared to the control treatment. In particular, L-leucine and L-phenylalanine promoted significant positive increases in seedling length (P-value < 0.05 in R). L-aspartic acid, L-cysteine, L-serine and L-tryptophan exhibited very severe (>80%) growth reductions in the presence of 60mM NaCl and 1mM L-amino acid (Figure 7).

Hypocotyl length was relatively unaffected by positive L-amino acid treatments. Even in treatments exhibiting significant increases in overall seedling length (L-leucine and L-phenylalanine) hypocotyl length remained similar to control treated seedlings (Figure 8). Treatments exhibiting substantial growth reductions did cause noticeable reduction in hypocotyl length. In particular, L-aspartic acid, L-cysteine and L-tryptophan exhibited no discernible hypocotyl development.

Radicle growth typically showed increased changes compared to control. All increases in total seedling growth over control occurred almost entirely in the radicle. Almost all treatments that exhibited reductions in overall seedling growth also caused radicle growth to decline. The sole exception to this was L-proline, which caused reductions in hypocotyl growth, but no change in

total seedling length as a result of increasing radicle growth (Figure 8).

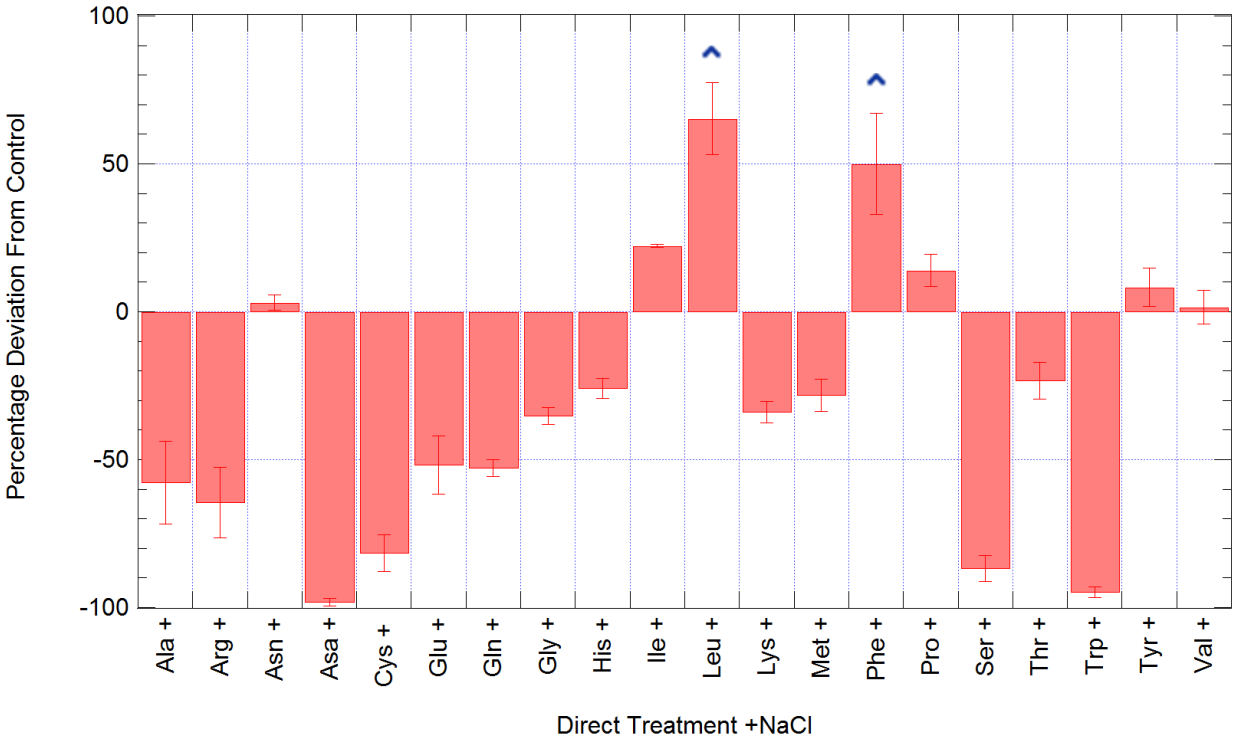

Figure 7: Seedling length following 48 hours direct treatment with each L-amino acid in the presence of 60mM NaCl, shown as percentage deviation from seedlings grown in 60mM NaCl solution alone. Error bars signify standard error of the mean. Statistically significant results are designated by blue arrows.

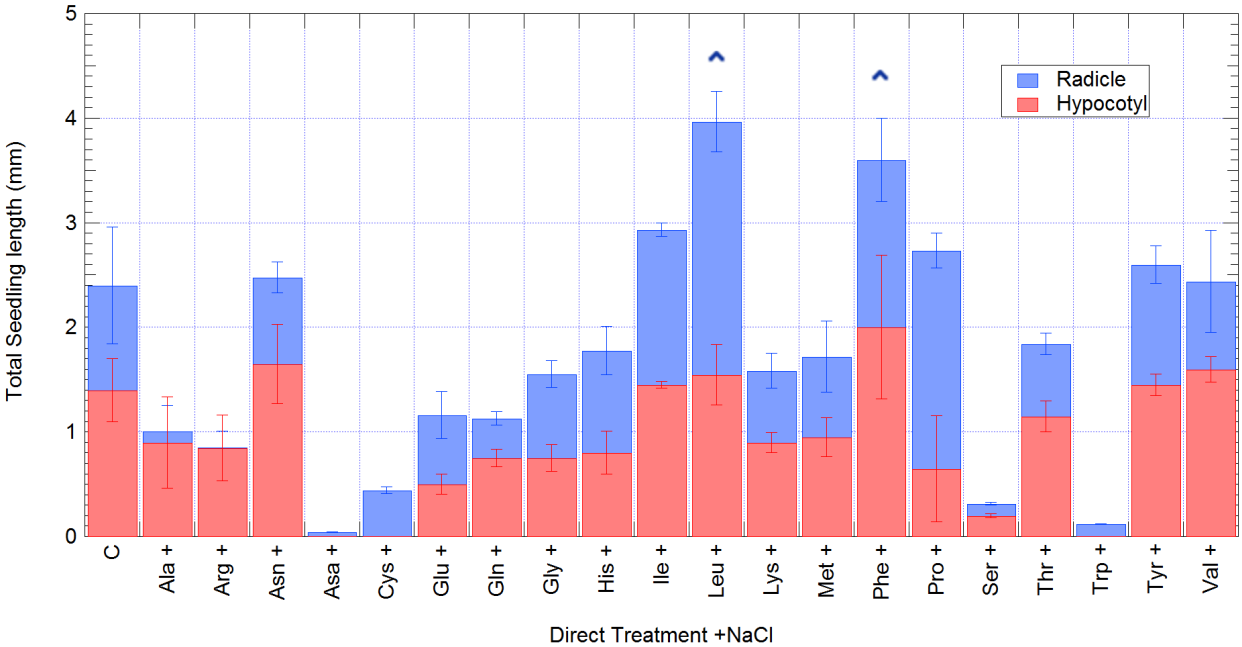

Figure 8: Hypocotyl and radicle lengths following 48 hours direct treatment with each of the 20 L-amino acids,or control, in the presence of 60mM NaCl. Error bars indicate standard error of the mean. Statistically significant results are designated by blue arrows.

3c. Pre-treatment with Amino Acids

Pre-treatment again showed a range of responses to different exogenous amino acids. There were also quite different results between pre-treatment and direct treatment experiments. The majority of treatments, however, still exhibited a negative effect on seedling length compared with the de-ionised water control. In general, reduction in growth was less severe than with direct treatments, however L-tryptophan treatment still reduced growth by over 50% (Figure 9). L-aspartic acid and L-isoleucine both had a non-significant positive effect on seedling growth after 48 hours. Treatment with L-valine caused a significant increase in growth over control-treated seedlings.

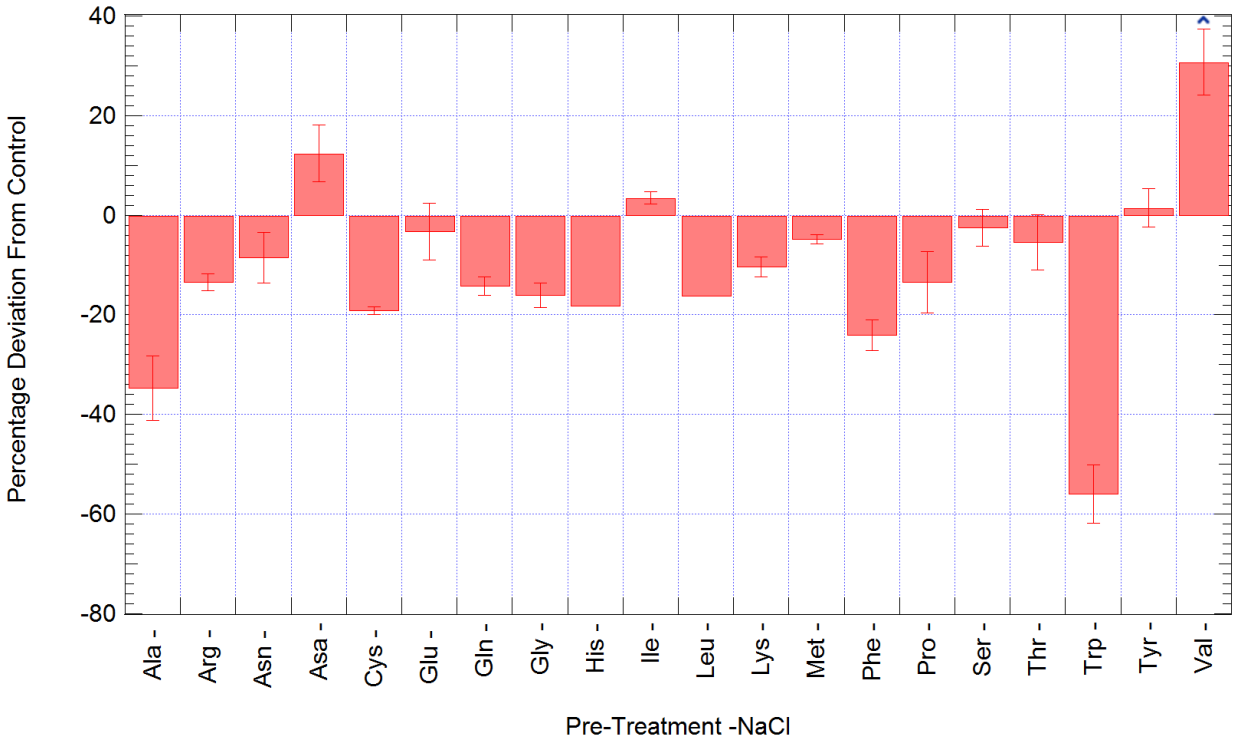

Figure 9: Seedling length following 8 hours treatment with each L-amino acid, then 40 hours in de-ionised water, compared to 48 hours growth in de-ionised water alone. Error bars indicate standard error of the mean. Statistically significant results are designated by the blue arrow.

Hypocotyl length was relatively constant between treatments, although L-glutamate and L-tyrosine exhibited noticeable increases in hyopcotyl length compared to control, without a corresponding increase in total seedling length (Figure 10). As with direct treatment results, the majority of total seedling length changes occurred in the radicle. This is particularly evident in L-valine treated seedlings, which had a very slight effect on hypocotyl length, but significantly increased total seedling growth via increased radicle development.

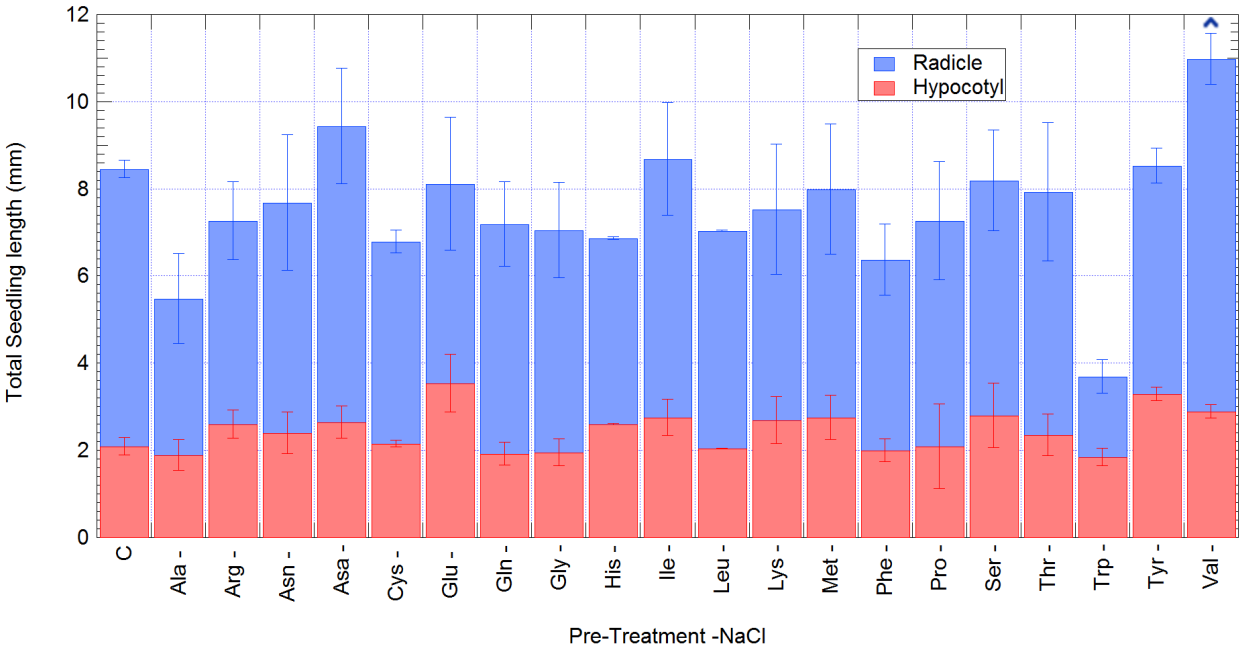

Figure 10: Hypocotyl and radicle lengths following 8 hours treatment with each L-amino acid, then 40 hours in de-ionised water, or 48 hours growth in de-ionised water alone. Error bars indicate standard error of the mean. Statistically significant results are designated by the blue arrow.

Seedling growth in the presence of 60mM NaCl was significantly positively affected by pre-treatment with several L-amino acids, compared to pre-treatment with de-ionised water only (Figure 11).

In the presence of 60 mM NaCl, the following amino acids resulted in about 40% or greater increase in seedling length: arginine, glycine, histidine, methionine and phenylalanine. There was generally less variation in seedling size between treatments than in direct treated or non-NaCl treated experiments (Figure 11). All but one (L-cysteine) of the non-significant treatments caused a less than 20% change in seedling growth compared to control. Contradictory to the other experiments, the majority of L-amino acid pre-treatments caused a positive effect on stressed seedling growth, compared to de-ionised water pre-treatment (Figure 9). The only treatments to have a negative effect on seedling growth were L-alanine, L-asparigine, L-glutamate and L-proline, none of which were statistically significant changes.

Hypocotyl growth showed slightly more variation in the presence of 60mM NaCl than when exposed to L-amino acids alone. In particular, significant changes to total seedling growth were more consistently associated with increases in hypocotyl length (Figure 12).

However, the majority of growth difference still occurred on the radicle, with changes in hypocotyl growth being relatively slight. An exception to this was L-tyrosine, which exhibited substantial increases in hypocotyl growth, but severely limited radicle development (Figure 12).

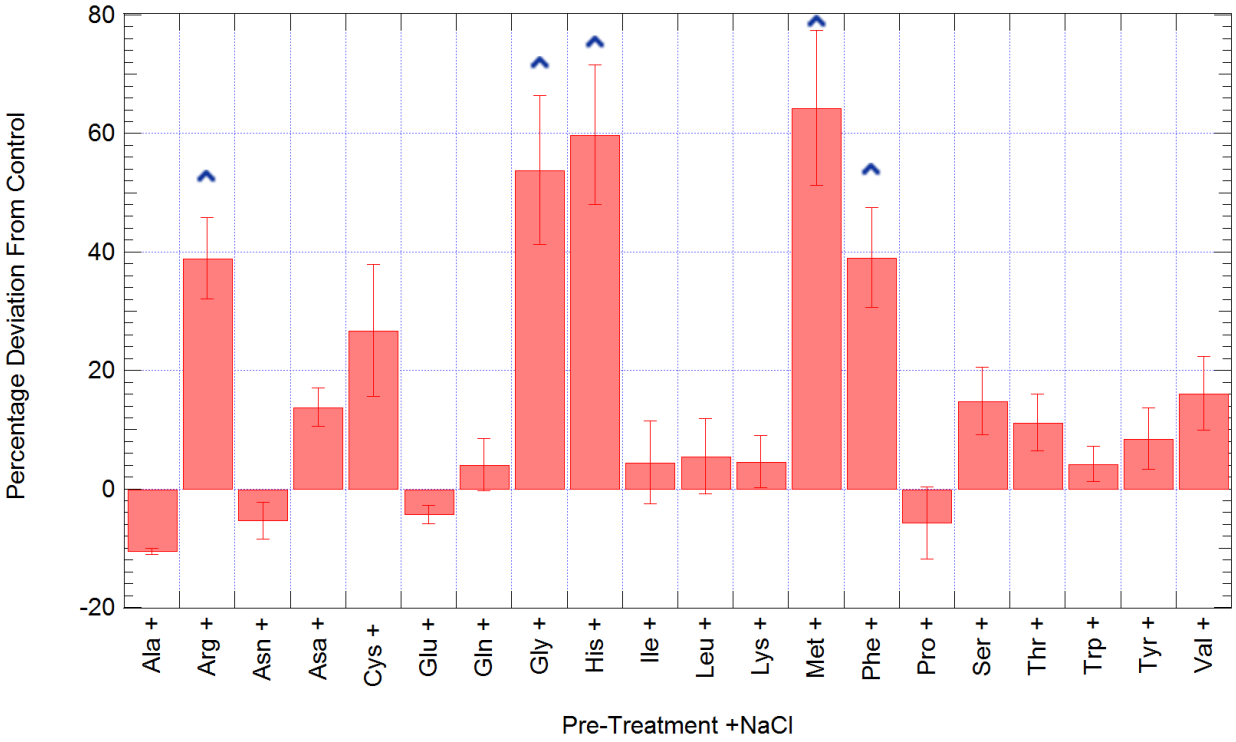

Figure 11: Seedling length following 8 hours pre-treatment with each of the 20 L-amino acids and 40 hours growth in 60mM NaCl, compared to pre-treatment with de-ionised water alone. Error bars indicate standard error of the mean. Statistically significant results are designated by blue arrows.

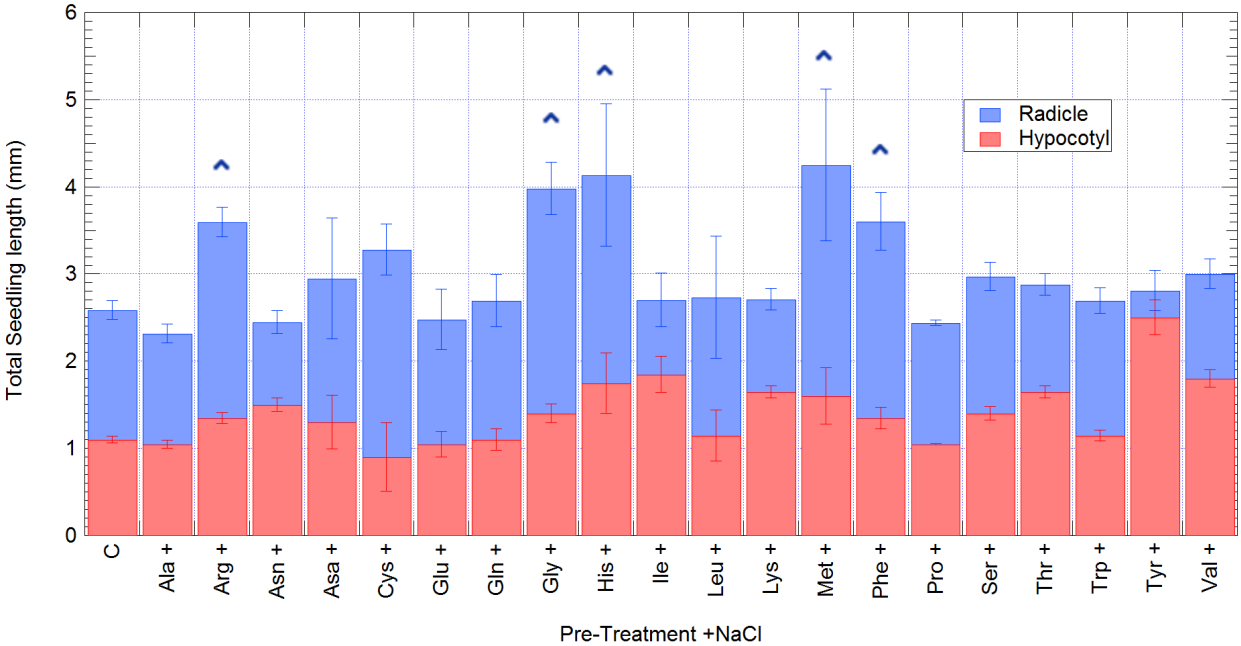

Figure 12: Hypocotyl and radicle lengths of seedlings following 8 hours L-amino acid or de-ionised water (control) pre-treatment and 40 hours growth in 60mM NaCl solution. Error bars indicate standard error of the mean. Statistically significant results are designated by blue arrows.

3d. Treatment under controlled pH conditions

The experiments with exogenous amino acids on lettuce seed germination and early seedling growth were repeated in MES buffer (pH 5.6) containing the amino acids and/or NaCl for comparison with the results from the previous experiments in which deionised water-based test solutions were used. Direct treatment in the absence of NaCl produced no significant impact on seedling growth. In the presence of NaCl, the same results were obtained whether the pH of the test solutions was adjusted to pH 5.6 or not (Table 2). Leucine and phenylalanine both exhibited a significant positive difference in growth compared to the control, based on 95% confidence intervals in R.

Table 2: Seedling growth in millimetres following treatment with L-amino acids of interest at a controlled pH of 5.6. Standard error of the mean in shown in brackets.

	Direct Treatment				Pre-Treatment						
	C	Ile	Leu	Phe	C	Arg	Gly	His	Met	Phe	Asn
+NaCl	1.25 (0.07)	1.42 (0.06)	1.65 (0.1)	1.86 (0.09)	1.86 (0.08)	2.61 (0.1)	2.82 (0.09)	1.97 (0.1)	3.08 (0.12)	2.58 (0.1)	1.99 (0.22)
Hypo	0.6 (0.02)	0.7 (0.04)	0.8 (0.04)	0.9 (0.04)	0.78 (0.06)	0.87 (0.05)	0.84 (0.04)	1.02 (0.08)	1.0 (0.05)	0.84 (0.06)	0.97 (0.14)

Seeds pre-treated at pH 5.6 and germinated in 60 mM NaCl mostly showed similar trends compared to non-pH adjusted treatments. Most amino acids tested exhibited a decrease in growth of ~30% compared to non-pH adjusted experiments. However, this was also exhibited in control treatments, therefore positive growth effects of most amino acid treatments were maintained (Table 2). There were two notable exceptions to this. Seedlings pre-treated with L-histidine at pH 5.6 exhibited a significant reduction in growth compared to the control, contradictory to what was found with the non-pH regulated treatment. Another notable exception was that the pre-treatment with L-asparagine in a pH-regulated environment caused a significant increase in growth compared to control, whereas in a non-pH controlled environment it did not. Hypocotyl growth was found to decrease at a greater rate than total seedling growth in pH controlled treatments, so that the hypocotyl was a smaller proportion of total seedling length than in non-controlled seedlings.

3e. Amino acids in combination

Seedlings treated with multiple L-amino acids in combination showed no positive effects compared to control. In the absence of NaCl, L-isoleucine/L-phenylalanine and L-leucine/L-phenylalanine treated seedlings exhibited increased growth compared to the treatments with the respective L-amino acids alone. However, growth in both cases was still substantially reduced compared to control. Treatment with all 3 L-amino acids tested produced the least seedling growth of all combinations (Table 3).
In the presence of 60mM NaCl, all L-amino acid combinations (L-ile/L-leu, L-ile/L-phe, L-leu/L-phe and L-ile/L-leu/L-phe) produced reduced growth compared to all 3 constituent L-amino acid treatments. L-ile/L-leu treatment exhibited non-significant (95% confidence interval in R) seedling growth promotion compared to control (60mM NaCl solution alone), however all other combinations resulted in reduced growth from control treatments.

Table 3: Total seedling length and hypocotyl length (mm) following 48 hours growth in 1mM of combined L-amino acids of interest, contrasted with growth following treatment with a sole target L-amino acid.

	C	Ile	Leu	Phe	Ile/Leu	Ile/Phe	Leu/Phe	Ile/Leu/Phe
-NaCl	8.65	7.17	5.28	4.48	5.97	7.95	6.06	5.88
Hypo	2.31	2.8	2.55	2.3	2.26	3.17	2.33	2.36
+NaCl	3.15	2.94	3.97	3.6	2.72	2.03	2.03	1.9
Hypo	2.03	1.45	1.55	2	1.29	1.17	1	0.92

3f. Cell size

Direct treatment
In lettuce seeds following 48 hours exposure to 60mM NaCl, in the absence of any exogenous L-amino acids, cells in the root maturation zone exhibited severely reduced length, but almost twice the width of those in seedlings exposed to de-ionised water only (Table 4). In the absence of NaCl, exogenous phenylalanine significantly reduced cell length but increased cell width compared to control without any exogenous amino acid. The other three amino acids of interest, L-isoleucine, L-leucine and L-valine had little effect on cell length but significantly increased cell width. However, in the presence of 60 mM NaCl, L-leucine and L-valine treatment caused a significant increase in cell length over control, but no significant change in cell width (95%

confidence interval in R). Treatment with L-phenylalanine also caused a significant increase in cell length, but this corresponded with a reduction in cell width. L-isoleucine caused no significant changes in either length or width from control (Table 4).

Table 4: Cell lengths and widths in the maturation zone of lettuce seedlings following 48 hours growth in; 1mM of an L-amino acid of interest, or de-ionised water, or 60mM NaCl solution, or 60mM NaCl accompanied by 1mM of an L-amino acid of interest. Standard error of the mean is shown in brackets.

DT	-NaCl					+NaCl				
	C	Ile	Leu	Phe	Val	C	Ile	Leu	Phe	Val
Length	53.44 (2.1)	52.83 (1.9)	55.21 (2.5)	31.07 (2.3)	57.08 (1.1)	16.17 (0.7)	17.23 (0.6)	22.03 (1.3)	24.52 (0.2)	23.25 (1.5)
Width	5.04 (0.2)	6.82 (0.3)	6.7 (0.6)	7.42 (0.1)	7.48 (0.7)	8.31 (0.4)	8.0 (0.2)	9.1 (0.2)	6.71 (0.4)	8.01 (0.3)

Pre-treatment

Seedlings from pre-treatment showed no significant (95% confidence interval in R) variation from control in cell width, either in stressed or unstressed conditions (Table 5).

However, a number of treatments exhibited significant changes in cell length. In unstressed conditions, seedlings treated with L-asparagine and L-threonine exhibited significantly increased average cell length compared to control treated seedlings.

In the presence of NaCl, seedlings from pre-treating seeds with asparigine, aspartate, glycine, histidine, isoleucine, or threonine all exhibited significantly lower cell length than control seedlings

Table 5a: Cell length and width in the maturation zone of lettuce seedlings following 8 hours treatment with an L-amino acid of interest, and 40 hours growth in de-ionised water.. Standard error of the mean is shown in brackets.

PT	-NaCl								
	C	Arg	Asn	Gly	His	Met	Phe	Thr	Val
Length	49.3 (2.0)	48.5 (1.7)	58.6 (0.8)	51.6 (1.6)	50.2 (2.1)	54.7 (2.1)	55.3 (2.0)	59.0 (0.9)	55.9 (1.0)
Width	6.86 (0.3)	6.96 (0.4)	6.64 (0.2)	7.47 (0.6)	6.39 (0.2)	7.11 (0.5)	6.28 (0.2)	7.2 (0.3)	6.47 (0.2)

Table 5b: Cell length and width in the maturation zone of lettuce seedlings following 8 hours treatment with an L-amino acid of interest, and 40 hours growth in 60mM NaCl solution. Standard error of the mean is shown in brackets.

PT	+NaCl										
	C	Arg	Asa	Asn	Gly	His	Ile	Met	Phe	Thr	Val
Length	33.2 (2.2)	30.9 (1.4)	23.3 (0.9)	22.5 (1.0)	24.6 (0.9)	25.6 (1.7)	24.1 (0.5)	26.3 (1.4)	27.5 (1.3)	24.0 (0.5)	29.5 (0.7)
Width	9.35 (0.2)	8.43 (0.7)	8.57 (0.3)	8.84 (0.2)	8.65 (0.4)	8.3 (0.7)	8.13 (0.3)	8.67 (0.9)	8.99 (0.3)	8.75 (0.3)	9.67 (0.3)

3g. Mitotic Activity

Direct treatment

In the absence of exogenous amino acids, 60 mM NaCl reduced mitotic activity in the root tip of lettuce seedlings compared to the deionised water control 48 hours after sowing (Table 6). In the presence of L-leucine but without NaCl, cell division in the root tip was similarly reduced compared to the deionised water control. In the presence of L-isoleucine, L-phenylalanine or L-valine, the reduction in mitotic index was less severe compared to that in the direct L-leucine treatment (Table 6).

In the presence of 60 mM NaCl, cell division in the root tips of seedlings following 48 hours direct treatment with L-isoleucine or L-phenylalanine was reduced to approximately half that in control (60mM NaCl only) seedlings. L-isoleucine and L-valine in the presence of NaCl caused no significant change in cell division rate (Table 6).

Table 6: Dividing cells per 1250 in the root tip of lettuce seedlings following 48 hours treatment with an L-amino acid of interest, or de-ionised water. Standard error of the mean is shown in brackets.

DT	C	Ile	Leu	Phe	Val
-NaCl	19.13 (2.1)	14.78 (1.2)	8.88 (1.0)	11.62 (1.0)	14.4 (1.2)
+NaCl	9.4 (1.5)	4.33 (0.5)	8.5 (0.8)	5.1 (0.4)	9.8 (0.7)

Pre-treatment

Cell division in the seedlings in deionised water control or in 60 mM NaCl did not show significant difference in pre-treated seedlings as well as those direct treated. An exception was that cell division in the seedlings pre-treated with L-glycine was about half the level in the deionised water control without any exogenous amino acid. None of the L-amino acids of interest exhibited significant differences in mitotic activity from control (Table 7).

Table 7: Dividing cells per 1250 in the root tip of lettuce seedlings following 8 hours treatment with an L-amino acid of interest, or de-ionised water, then 40 hours growth in either de-ionised water or 60mM NaCl solution.

PT	C	Arg	Gly	His	Met	Phe	Val
-NaCl	11.8 (1.0)		5.7 (0.4)				8.9 (0.6)
+NaCl	7.8 (0.9)	7.3 (0.5)	8.7 (0.9)	7.1 (0.7)	8.6 (0.8)	8.18 (0.9)	8.15 (0.7)

Discussion

4a. Selection of NaCl and amino acid concentrations for the main investigations in this work

The initial experiment on the different concentrations of NaCl on lettuce seed germination and seedling growth showed a clear increase in the severity of the negative effect on these two processes as NaCl concentration was increased. The NaCl concentration chosen for the majority of the experiments here was 60 mM, as it produced a significant reduction in seedling growth, while not seriously affecting the germination rate. Higher concentrations (90 mM and above) resulted in severely reduced germination rates, reducing the usable sample of seedlings produced per experiment.

During initial preliminary experiments to identify general trends, L-amino acids at concentrations of 1 mM were found to produce noticeable differences in seedling growth between treatments. At lower concentrations of amino acids often no effect was observed, and at higher concentrations almost all L-amino acids caused general reductions in seedling growth. Since distinct identification of differences in growth effect between treatments with different amino acids at 1 mM was possible, this concentration was chosen for further investigations in the primary objective of the present research.

4b. Comparison of direct and pre-treatment with amino acids in the absence of NaCl

When directly treated for 48 hours, all amino acids tested had a negative impact on seedling growth compared to de-ionised water (control) treatment. These results, which were obtained from seedlings exposed to L-amino acids but no salinity stress, demonstrate that all of the L-amino acids could operate as a stress under normal conditions. The findings are consistent with some but not all conclusions drawn in the previous studies. A study on the cell suspension cultures of *Nicotiana sylvestris* showed that a few exogenous amino acids added to culture media were highly detrimental to cell growth and development (Bonner et al. 1996). In this study, it was also found that L-glutamine exhibited a powerful positive growth effect under normal conditions, and in the presence of several other amino acids. This exception was not found in the current study; all 20 L-amino acids had a toxic effect on seedling growth. This discrepancy may be related to species, or growth stage differences.

The finding of the growth inhibitory effect of amino acids in the present study is also different

from more recently published studies which suggest that a number of exogenously applied L-amino acids have a positive effect on seedling growth through nutritive benefits. In particular, Svennestram et al. have explored the impact of amino acids, both L- and D- enantiomers, as secondary nitrogen sources (Forsum et al. 2008; Svennerstam, et al. 2008). These experiments identified several amino acids that promoted growth in *Arabidopsis* by acting as a nitrogen source. In particular, L-glutamine and L-asparigine were identified as the most effective in promoting plant growth. These experiments also highlighted several amino acids that had a detrimental effect on plant growth, including L-isoleucine and L-valine as identified in earlier research (Bonner and Jensen 1997).

In the pre-treatment experiments, the majority of L-amino acids had a detrimental effect on seedling growth. However, reductions in growth compared to control (pre-treatment with deionised water) were generally less severe than seedlings in the direct treatments. In the pre-treatment, while a few L-amino acids (L-aspartatic acid, L-isoleucine and L-tyrosine) did not inhibit seedling growth compared to control (pre-treatment with deionised water), only L-valine provided a significant increase in growth compared to the control. These results from the pre-treatment of the 20 amino acids further suggest that most amino acids are inhibitory to lettuce seedling growth under normal conditions.

The reduced severity of this effect for most of the L-amino acids in the pre-treatment compared to direct treatment is likely due to a shortened exposure time. The full 48 hours of exposure in the direct treatment experiment allows any toxic effects to have a far greater impact than in seedlings exposed for only 8 hours as in the pre-treatment experiments.

Different treatment methods for a few L-amino acids clearly operated via different mechanisms, however. In particular, the direct and pre-treatment method with L-asparagine and L-valine resulted in opposing responses. In the direct treatment experiments, both of these L-amino acids had extremely detrimental effects on seedling growth, causing reductions, 89.9% and 41.6% respectively, in growth compared to the control (sowing seeds in deionised water in the absence of any exogenous amino acids). In pre-treatment experiments, however, both L-asparagine and L-valine were found to promote seedling growth compared to the control (pre-treatment with deionised water). This clearly suggests a different mechanism of action for some of the L-amino acids between direct / continuous treatment and pre-treatment. This is a novel finding worthy of further investigations to understand the precise nature of the mechanisms involved.

4c. Alleviation of salinity stress

Many L-amino acids exhibited significant salinity stress alleviation, both in direct treatment and pre-treatment experiments. This shows a clear mechanism of specific stress relief, rather than general growth increase. L-valine was the only L-amino acid to provide a general growth response as well as reducing the impact of salinity stress on seedling growth. This suggests a specific response arising from the interaction of these L-amino acids and the NaCl that did not occur in the presence of either stressor alone.

In the pre-treatment experiments, several L-amino acids were shown to be able to alleviate the negative growth impact of high salinity stress. This response is most likely the result of a priming effect on induction or activation of plant defences or tolerance mechanisms by the effective L-amino acids similar to other pre-sowing seed priming studies using a variety of priming agents including sub-lethal levels of NaCl stress (Jisha et al. 2012). Priming-induced responses have been considered as plant mechanisms for defence against herbivore, pathogen damage and abiotic stress. Diverse mechanisms might be involved so that an initial attack or environmental cue could prompt physiological changes in primed seeds to better prepare for subsequent biotic attacks or abiotic stress (Frost et al. 2008; Jisha et al. 2012).

In the present study, L-amino acids which successfully alleviated salinity damage are likely to operate in a similar way to other seed priming agents. The pre-germination exposure to selected amino acids could prompt the activation of defence mechanisms to protect against the toxic effect of the L-amino acids in question. In addition, following germination, these already activated defences would continue to protect the seedlings from salinity stress while seeds not pre-treated with just deionised water did not have this protection.

It has been shown that salinity stress often adversely affects water uptake (osmotic stress) (Almansouri et al. 2001) and induces oxidative stress (production of reactive oxygen species or ROS such as hydrogen peroxide, lipid hydroperoxide, etc.)(Afzal et al. 2013). Therefore, salinity stress tolerance in plants would involve physiological adjustment for increased water uptake including an increase in solute concentration such as L-proline and / or antioxidative defence (Deinlein et al. 2014). It could be speculated that amino acids and other priming agents that have an alleviative effect against salinity stress during early seedling growth might also have an effect on these salinity tolerance mechanisms.
Some previous studies have suggested that exogenous proline can have a negative impact on

plant growth under non-stressful conditions (Szabados and Savoure, 2010). More recent papers are generally in agreement that there are positive growth effects of L-proline on plants under stress(Kavi Kishor 1995; Hong 2000; Kaul et al. 2008; Nawaz et al. 2010) . In contrast, the present study found that proline had no positive effect on growth, either in stressed or unstressed conditions. This was found in both direct and pre-treatment experiments.

L-glutamate is another L-amino acid that has been previously found to alleviate salt stress on cucumber seed germination (Chang et al. 2010). This positive effect of L-glutamate was not observed in pre-treatment of lettuce seeds with this amino acid in this study. In direct treatment experiments, L-glutamate consistently caused significant reductions in seedling growth compared to that of control (deionised water without any exogenous amino acids).

The differences in findings between this study and those of other studies may be due to the use of different plant species in these experiments. This highlights the need for further research in this field. The effect and use of each of the L-amino acids is influenced by a wide range of factors, including species, stage of life and presence of other abiotic stressors.

The differences in the effects between direct treatment and pre-treatment with amino acids clearly show that exposure to many L-amino acids before germination can continue to have significant positive effects on growth and development during and after germination. This provides the opportunity for these to be used to enhance seed vigour and increase the health of crop plants through treatment before sowing. In addition, based on the amino acid pre-treatment results obtained, it is suggested that the amino acids identified to have an alleviative effect against salt stress are potentially new useful seed priming agents. Further research in this area, and with seeds of species besides lettuce, would be worthwhile to further develop and validate this discovery.

In preliminary experiments conducted, lettuce seeds pre-treated before germination with selected amino acids were first air-dried for 12 hours before being sown on deionised water in the absence or presence of NaCl stress. It was found that the beneficial effects of the pre-treatment with amino acids were retained even when seeds were dried before germination occurred (results not shown). Further work validating this should lead to the development of practical methods for the use of L-amino acids as seed priming agents to promote seedling growth, and protect against salinity stress.

Many of the results obtained suggest a different mechanism of action between direct treatment and pre-treatment with amino acids regarding alleviation of salinity stress on seedling growth. As mentioned earlier, this may be partly attributable to an exposure and dosage effect. However, many of the results suggest more fundamental differences in action between the two treatment types. For example, in stressed seedlings, L-leucine was capable of significant alleviation of salinity stress, but when used to pre-treat seeds, it had no notable effect on seedling growth compared to control (pre-treatment with deionised water only). This effect is also noticeable with L-isoleucine and L-valine, although to a lesser degree. Apart from these three amino acids, all other L-amino acids had a more positive or statistically identical effect on seedling growth when the respective pre-treatment and direct treatment experiments were compared. This suggests different mechanisms of action not only between treatment methods, but also between the different L-amino acids.

The results of this study highlight the complexity of the stress response activity of the L-amino acids; no single L-amino acid exhibited a significant positive growth effect in all four basic treatment types. Only a single L-amino acid, L-phenylalanine, was favourable for both direct treated and pre-treated seedlings in any of the experiments. This, again, demonstrates that there appear to be a huge range of factors, and separate mechanisms which could be employed by different L-amino acids to influence seedling growth in different growth conditions.

4d. pH effect

The results of the direct and pre-treatment experiments using MES buffer (pH 5.6)-based test solutions containing amino acids largely confirmed that the observed amino acid effects in the present study were not related to variation in pH of the test solutions in most treatments except L-histidine and L-asparagine.

4e. Cellular growth mechanisms

From the seedling length measurements, salinity stress or exogenous amino acids in the absence of NaCl mainly affected root growth. Therefore, it was necessary to examine the cellular basis of root growth. Two of the many basic developmental processes related to root cell growth (Ding et al. 2010), cell division in the root tip and cell elongation in the maturation zone of lettuce roots (radicles), were investigated in the present study. This was to determine if the effect of exogenous amino acids on root growth in the absence or presence of 60mM NaCl was related to

these two basic cellular processes.

Examination of changes in cell growth was generally inconclusive, but did reveal some intriguing results. Cell length was increased over control in many situations, most consistently in seedlings under salt stress in direct treatment experiments.

Cell length

There were some interesting changes in cell length between treatments. In particular, seedlings in the direct treatment in the absence of NaCl developed noticeable increases in cell width in the presence of all L-amino acids investigated. Surprisingly, in this case, only L-phenylalanine exhibited a substantially negative effect on cell length in normal conditions. Despite all L-amino acids significantly reducing unstressed seedling growth, none of the other L-amino acids investigated had any effect on cell length in the maturation zone of the root. This difference may be the result of different mechanisms of action between L-phenyalanine and the other L-amino acids investigated in this experiment. Further study in this area is required, and may produce useful conclusions regarding the action of different exogenous L-amino acids at the cellular level. Overall, cell length did not seem to have been consistently affected by L-amino acid treatment.

Changes in cell width are often associated with increased ethylene production, which is believed to play an integral role in plant stress responses (Morgan and Drew 1997; Zapata et al. 2003). The effect of this response to salinity stress can be clearly seen here. In both direct treatment and pre-treatment experiments, salt-treated seedlings exhibited substantially increased cell width in all but two cases. The exceptions to this were seedlings in the direct treatment with L-leucine or L-phenylalanine. In these cases cell width was practically identical between -NaCl and +NaCl treated seedlings. Thus, L-leucine and L-phenylalanine were the only L-amino acids that provided significant salinity stress protection in the direct treatment experiment, suggesting an interaction of L-leucine and L-phenylalanine with ethylene as part of the salinity stress response. In cucumber, it was L-glutamate that alleviated salt stress on seed germination and exhibited an interaction with ethylene (Chang et al. 2010). Therefore, it is possible that L-leucine and L-phenylalanine could also interact with ethylene in the same way to influence lettuce seed germination and seedling growth. In unstressed conditions, all of the investigated L-amino acids caused a noticeable increase in cell width compared to control treated seedlings.
Some amino acids have previously been identified as promoters of ethylene production (Matilla 2000). The effect of this relationship on plant physiology is relatively unknown, however. The

results obtained in this experiment suggest an intrinsic relationship among L-amino acid supply, and hormonal and physiological changes to seedlings. Pre-treatment results suggest that this effect is reliant upon continued, or at least post-germination exposure.

Mitotic Activity

Most direct treatment with amino acids produced significant reductions in mitotic activity compared to control seedlings (not exposed to amino acids). The only direct treatment that did not reduce mitotic activity was L-valine +NaCl, which produced an almost identical rate of cell division to control seedlings. In unstressed conditions, L-amino acids appeared to have a strong negative influence on mitotic activity. This effect was found in both direct treatment and pre-treatment with all investigated L-amino acids, suggesting a common mechanism of amino acid toxicity. Reductions in the cell division rate were evident even in seedlings pre-treated with L-valine which promoted seedling growth overall.

Seedlings pre-treated with amino acids and germinated under NaCl stress conditions, however, exhibited no practical difference in cell division rates from control (pre-treated with deionised water). This is an additional evidence of an interaction between priming with amino acid and salinity stress.

Both salinity stress and exogenous L-amino acids had a noticeable effect on both cell growth and cell division rates. However, neither of their effects at the cellular level seemed to be of such a magnitude, or with a consistency that could explain the effects on seedling growth that were observed in many treatments. This leaves the method of action for the beneficial L-amino acids unclear. More in-depth study into the effect of L-amino acids on cell elongation and division is required to form a comprehensive understanding of the way L-amino acids are able to benefit post-germinative seedling growth.

4f. Conclusions

In conclusion, this study has identified interaction between L-amino acids and salinity tolerance during and immediately after seed germination which have not been identified in current published literature. In particular, pre-germination application of several L-amino acids; L-arginine, L-glycine, L-histidine, L-methionine and L-phenylalanine, were found to induce a priming stress response that promotes resistance to salinity stresses post-germination. The present findings have added some new information, and suggested avenues for follow-up research which may yield beneficial information about plant stress responses during seed germination and early post-germinative seedling growth. These findings suggest potential commercial applications of several L-amino acids in growth promotion and salt alleviation in developing seedlings. Identification of a toxic effect from all protein amino acids in unstressed plants contributes to the controversial discussion on plant utilisation of exogenous amino acids. The differences in response identified between treatments (direct vs. pre-treated and -NaCl vs +NaCl) highlight the complexity of the relationship between amino acids and plant growth, as well as the many factors which can influence this relationship. Further study into these relationships may enable more consistent practical utilisation of exogenous amino acids to enhance plant growth.

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